Human Liver High Molecular Weight Zinc-Dependent Acid p-Nitrophenylphosphatase. Purification and Properties

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Human liver contains high molecular weight-type Zn\(^{2+}\)-dependent acid p-nitrophenylphosphatase (HMW-ZnAP). The enzyme was purified 1000-fold by a new procedure, including preparative isoelectrofocusing. The HMW-ZnAP was homogeneous in non-denaturing disk-gel electrophoresis with an MW of about 93 kDa determined by Sephadex G-100 chromatography. A single polypeptide chain of 43 kDa was detected on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), suggesting a homodimeric structure. The isoelectric point (pI) was 7.2—7.4. Human liver HMW-ZnAP requires Zn\(^{2+}\)-ions for activity; other divalent cations are ineffective or act as inhibitors. It dephosphorylated p-nitrophenylphosphate (pNPP) (K\(_m\) = 0.24 mM), o-carboxyphenolphosphate (oCPP) (K\(_m\) = 0.92 mM) and phenylphosphate (PhP) (K\(_m\) = 1.42 mM). Other substrates including \([\text{P}^{32}\text{P}]\)-labelled casein or phosvitin, adeny1 nucleotides and myo-inositol-1-phosphate, were not dephosphorylated. Human liver HMW-ZnAP obeys Michaelis-Menten kinetics with pNPP as substrate; the enzyme was competitively inhibited by inorganic phosphate (K\(_i\) = 0.55 mM), and by oCPP (K\(_i\) = 0.65 mM) and PhP (K\(_i\) = 1.16 mM). Adenosine monophosphate (AMP), adenosine diphosphate (ADP) and ATP displayed mixed-type inhibition. The enzyme was also inhibited by some modifiers such as EDTA, oxalate, p-chloromercuri-benzozoate, trastate,imidazole, cyanide, cysteine, histidine and diethylpyrocarbonate, but not by fluoride or okadaic acid. Human liver HMW-ZnAP is sensitive to temperatures higher than 40 °C. The pH-dependence of the steady-state kinetic parameters indicates the existence of an essential ionizable group with a pK\(_a\) of 7.25—7.50, similar to that of histidine. However, diethylpyrocarbonate inactivation experiments suggest that other amino acid residues may also be involved in enzyme catalysis.

Key words zinc-dependent acid phosphatase; enzyme purification; kinetic study; diethylpyrocarbonate inactivation; human liver

Protein phosphorylation is a post-translational modification which plays a key role in signal transduction. The level of protein phosphorylation by specific protein kinases is controlled by removing the phosphoryl groups by phosphatases. The equilibrium between phosphorylation and dephosphorylation is one of the most important regulatory events in living cells. In the last ten years phosphoproteinphosphatase activity has been demonstrated for enzymes known as acid phosphatases and, in particular, prostatic and bone acid phosphatase.1,2 In vertebrate tissues, three main classes of acid phosphatase (AP, orthophosphoric monoester phosphohydrolase, acid optimum, C.3.1.3.2.), differing in relative molecular weight (MW) and indicated as high- (HMW-AP), intermediate- (IMW-AP) and low- (LMW-AP) MW AP have been described.3,4 The phosphotyrosylproteinphosphatase activity of IMW-AP has been reported4,5 and, recently, phosphotyrosylproteinphosphatase activity was also demonstrated for LMW-AP isolated from many organs and tissues of mammals,5,7 birds8 and amphibians.9 These findings suggest a new role for nonsyosomal APs in the control of cell proliferation, differentiation and transformation.

The presence of another AP class which hydrolyzes p-nitrophenyl phosphate (pNPP) in the presence of Zn\(^{2+}\) ions, called ZnAP, has been reported in mammalian tissues.10—14 The enzyme exists in two major forms: LMW-ZnAP, about 60 kDa, abundant in brain,11,12,14,15 testicular and prostatic tissues11; and HMW-ZnAP, about 100 kDa, characteristic of the liver, intestine and kidney.10—12 A partial characterization of the bovine liver enzyme has been previously published.10 The purification, as well as the biochemical and structural characterization of the bovine brain LMW-ZnAP, was also reported recently.13,14 Interestingly, the LMW enzyme shows both Zn\(^{2+}\)-dependent AP activity and Mg\(^{2+}\)-dependent myo-inositol-1-phosphate phosphatase activity.13,14 APs are widely distributed in human tissues2,3,16 and, in the liver, three APs were identified.17 Among these enzymes, the lysosomal HMW-AP and cytosolic LMW-AP were isolated and extensively characterized.18,19 To date, no information is available concerning the presence of zinc-ion dependent AP activity in human liver. In this paper we demonstrate the existence of an HMW-ZnAP type in human liver. The enzyme was purified using a different procedure to that reported for bovine liver,10 and was characterized with respect to MW, metal ion dependence, inhibition, pH optima, temperature sensitivity, kinetic properties and substrate specificity.

MATERIALS AND METHODS

Reagents Substrates, diethylpyrocarbonate (DEP), protein markers and cAMP-dependent protein kinase from porcine heart were purchased from Sigma Italia. [γ-32P]ATP was from Amersham (U.K.). Reagents for electrophoresis were obtained from Bio-Rad (U.S.A.). Amphotolines and Sephadex G-100 and G-200 from Pharmacia (Sweden). CM-cellulose was obtained from Whatman (U.K.). Calf thymus protein kinase CKII was prepared

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as described. All reagents were made with deionized water and water double distilled from glass.

**Enzyme Purification** The purification data relate to about 100 g tissue. All operations were carried out at 4 °C unless otherwise specified. Specimens of normal human liver, obtained 24 h after death, were homogenized in 0.3 M sodium acetate buffer (pH 6.0) and the homogenate centrifuged at 45000 × g for 90 min. The clear supernatant was fractionated using ammonium sulphate. The fraction obtained between 17—40% (w/v) was divided into two batches and each passed through a Sephadex G-200 column (3.5 × 60 cm) equilibrated with 25 mM sodium acetate buffer (pH 6), containing 150 mM NaCl. The fractions containing ZnAP activity were concentrated with 40% (w/v) ammonium sulphate, dialyzed against 10 mM sodium acetate buffer (pH 6), and then applied to a CM-cellulose column (2 × 15 cm) equilibrated with 10 mM sodium acetate buffer (pH 6). The elution was carried out with a linear NaCl gradient (0—0.35 M). The enzyme peak obtained between 200 and 250 mM NaCl was concentrated with ammonium sulphate, dialyzed against 10 mM sodium acetate buffer (pH 6.0), and subjected to preparative column isoelectric focusing (IEF) (LK B 8101 column, 110 ml). The IEF was carried out according to the manufacturer's instructions, at 4 °C with 1% (w/v) ampholines (pH 5—8 and 7—9) and at 450 volts for 48 h. Fractions (1 ml) were collected and pH values and enzyme activity assayed. The enzyme peak (about 10 ml), which eluted between pH 7.0—7.6, was divided into 5 aliquots and each chromatographed on a Sephadex G-100 column (1.8 × 85 cm), equilibrated and eluted with 50 mM sodium acetate buffer (pH 6) and 100 mM NaCl to remove glycerol and ampholines. The Sephadex G-100 column was also calibrated for MW determination. The final enzyme preparation was used for biochemical characterization. If necessary, the enzyme solution was further concentrated by readсорbing it onto a microcolumn (1 × 1 cm) of CM-cellulose and carrying out a single step elution with 0.5 M NaCl.

**Electrophoresis and Gel IEF** Discontinuous non-dissociating gel-electrophoresis was performed at 4 °C on 7.5% polyacrylamide rod gels. Proteins were stained with Coomassie blue. Gel IEF was performed as suggested by Drysdale et al. on 4% polyacrylamide gels containing ampholines in the pH range 5—8. After the run, the gels were sliced into 2 mm sections and eluted overnight in 150 μl 0.2 M acetate buffer (pH 5.8) to determine ZnAP activity. The pH profiles were obtained on duplicate gels, sliced and eluted in 1 ml distilled water. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% mini slab-gels with the following proteins as markers: α2-macroglobulin (170 KDa), phosphorylase B (97.4 KDa), glutamate dehydrogenase (55 KDa), lactate dehydrogenase (36 KDa) and trypsin inhibitor (20.1 KDa).

**Assay of ZnAP Activity** ZnAP was routinely assayed in 50 mM sodium acetate buffer (pH 5.8) containing 5 mM ZnCl₂ and 2.5 mM pNPP as substrate; the enzyme solution was in a final volume of 0.5 ml. After incubation at 37 °C for 5—10 min, the reaction was stopped by adding 1—2.5 ml 1 M NaOH and the extinction of p-nitrophenolate was read at 410 nm (ε = 1.8 × 10⁴ M⁻¹·cm⁻¹). One enzyme unit corresponds to 1 μmol p-nitrophenol liberated per min. Specific activity was expressed as units/mg protein.

The kinetic parameters, Kₘ and V, were calculated by nonlinear regression of the data. The values ± S.D. refer to two independent experiments carried out in triplicate. Ten substrate concentrations ranging from 0.2 to 5 × Kₘ were used. The inhibition constant (Kᵢ) of some compounds with respect to pNPP hydrolysis was determined graphically using the Lineaweaver-Burk plot and by replottting the slopes against the concentration of the inhibitor.

The effect of some modifier substances was tested at various concentrations of the inhibitor. After incubation of the enzyme with 50 mM acetate buffer, pH 5.8, and modifier at room temperature for 10 min, the residual activity was measured by adding 2.5 mM pNPP and 5 mM ZnCl₂. The values are reported as the concentration of the modifier that gave 50% enzyme inactivation (I₅₀). At least five different concentrations of each compound were tested.

Thermal stability was carried out by incubating the enzyme at 5 °C intervals from 20 to 50 °C in 50 mM Tris–acetate buffer (pH 7). The enzyme activity was determined as described above after the sample was kept in an ice-bath for 30 min.

The hydrolysis rate of various phosphate esters was carried out at 37 °C by measuring the liberated orthophosphate. All substrates were used at 5 mM final concentration with the exception of adenosine monophosphate (AMP) and myo-inositol-1-phosphate which were used at 2 mM.

The phosphoprotein phosphatase activity was assayed using casein and phosvitin phosphorylated by calf thymus protein kinase CKII or by the catalytic subunit of cAMP-dependent protein kinase from porcine heart as reported. The enzyme activity was determined in an incubation medium containing purified ZnAP, 3 mg/ml [³²P]-labeled casein, or phosvitin (about 70000 cpm), 5 mM ZnCl₂, 100 mM NaCl, 50 mM sodium acetate buffer (pH 5.8), or 50 mM Tris–acetate buffer (pH 7.2). After 10 min at 37 °C, the reaction was blocked with cold 5% trichloroacetic acid containing 1.5% pyrophosphate (TCA-pyrophosphate) and the solution was then filtered through 0.45 μm Millipore disks. After several washes with TCA-pyrophosphate, the residual radioactivity on the filter was determined from the Cerenkov count with respect to a control reaction mixture not containing ZnAP.

**Effect of pH on Kinetic Parameters and DEP Modification Studies** The pH-dependsence studies of the kinetic parameters for pNPP hydrolysis were carried out at 37 °C in 50 mM sodium acetate (pH 4.5—6.0), 50 mM dimethylglutarate–HCl (pH 5.8—7.0), 25 mM Tris–acetate (pH 6.5—7.5) and 50 mM Tris–HCl (pH 7.0—8.5). At each pH value, Kₘ and V were estimated as described above.

DEP inactivation studies were carried out as described by Miles in the presence or absence of 20 mM phosphate as protecting agent. Prior to each experiment, DEP was freshly diluted in cold ethanol and the concentration determined by reaction with imidazole.
unit was incubated with different DEP concentrations ranging from 0.4 to 4 mM, in 0.1 M sodium acetate buffer (pH 6.0). At the required times, aliquots were withdrawn and the residual activity assayed as reported above. Decarboxylation with 0.5 M hydroxylamine of the treated enzyme was monitored as described by Miles.

### RESULTS AND DISCUSSION

#### 1. Enzyme Purification

Preliminary attempts to purify the enzyme using the method reported for bovine liver were unsuccessful. In this new purification procedure the preparative IEF step (Fig. 1A) was crucial to obtain a homogeneous preparation of the human liver ZnAP, although the final enzyme activity and percentage recovery were lower than those obtained with bovine liver (Table 1). The enzyme was purified about 1000-fold and shows a specific activity of 8.9 units/mg protein. The recovery, although low, could be underestimated because of the presence in human liver homogenate of many pNPP hydrolyzing enzymes at pH 5.8-7.1. In addition, while LMW-AP was separated after Sephadex G-200 fractionation, the lysosomal HMW-AP and ZnAP were separated only after CM-cellulose chromatography. The final Sephadex G-100 chromatography gave a symmetrical peak of enzyme activity, coincident with the protein peak (Fig. 1B), which is homogeneous using both non-denaturing gel-electrophoresis (Fig. 2A) and SDS-PAGE (Fig. 2B). The native enzyme shows an MW of 93 kDa. The SDS-PAGE analysis shows a single band of 43 kDa suggesting that the human liver ZnAP is a homodimeric protein and seems to be an HMW-ZnAP-type. The pI determined with column and gel IEF was 7.4 and 7.2 respectively.

#### 2. Enzyme Properties

The native form of human liver HMW-ZnAP requires Zn$^{2+}$-ions for activity. The maximum enzyme activity was observed at 3-5 mM ZnCl$_2$ concentration. Other divalent cations including Mg$^{2+}$, Ca$^{2+}$, Ba$^{2+}$, Sr$^{2+}$, Mg$^{2+}$, Fe$^{3+}$, Al$^{3+}$, Cd$^{2+}$, Sn$^{2+}$, Pb$^{2+}$, Cu$^{2+}$, and Hg$^{2+}$, examined individually, failed to stimulate enzyme activity significantly and only Mn$^{2+}$ showed a slight activation (about 5-8% with respect to zinc ions).

Although a large number of substrates were assayed in our experiments, the physiological substrate of the enzyme is unknown. Human liver HMW-ZnAP efficiently hydrolyzes pNPP ($K_m = 0.24$ mM), o-carboxyl phenylphosphate (oCPP, $K_m = 0.92$ mM) and phenylphosphate (PhP, $K_m = 1.42$ mM) (Table 2). The phosphorylated amino acid phospho-L-serine (P-L-ser), was not hydrolyzed at an appreciable rate. The enzyme does not dephosphorylate a-casein, phosvitin or adenylic nucleotides. Similar results were reported for HMW-ZnAP of bovine liver. It has been reported that LMW-ZnAP, isolated from rat tissues and bovine brain, showed Mg$^{2+}$-dependent myo-inositol-1-phosphatase activity assayed at pH 7.4-8.0. The HMW-ZnAP from human liver showed no activity with myo-inositol-1-phosphate as substrate at pH 5.8 and 7.4, both in the absence and presence of Mg$^{2+}$ ions. All the phosphate esters tested, including β-glycerol phosphate, 2-naphthyl phosphate, βNADP and ATP, in

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Proteins (mg)</th>
<th>Activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>10500</td>
<td>95</td>
<td>0.009</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>2900</td>
<td>46</td>
<td>0.016</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>G-200</td>
<td>263</td>
<td>32</td>
<td>0.12</td>
<td>70</td>
<td>13</td>
</tr>
<tr>
<td>Cellulose-CM</td>
<td>18</td>
<td>17</td>
<td>0.95</td>
<td>37</td>
<td>106</td>
</tr>
<tr>
<td>IEF</td>
<td>0.46</td>
<td>2</td>
<td>4.3</td>
<td>4</td>
<td>478</td>
</tr>
<tr>
<td>G-100</td>
<td>0.09</td>
<td>0.8</td>
<td>8.9</td>
<td>2</td>
<td>989</td>
</tr>
</tbody>
</table>

The values were derived from processing 100 g tissue.

![Fig. 1. A. IEF Profile of Human Liver HMW-ZnAP from CM-Cellulose Chromatography](image)

![Fig. 2. A. Polyacrylamide Disc-Gel Electrophoresis of Purified Human Liver HMW-ZnAP (About 50 µg), Stained with Coomassie](image)
Table 2. Hydrolysis of Some Substrates by Human Liver HMW-ZnAP

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative enzyme activity (%)</th>
<th>$K_m$ (mm)</th>
<th>$V$</th>
<th>$I$</th>
<th>$K_i$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNPP</td>
<td>100</td>
<td>0.24±0.06</td>
<td>9.20±0.54</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>oCPP</td>
<td>72</td>
<td>0.92±0.12</td>
<td>7.40±0.60</td>
<td>C</td>
<td>0.65±0.09</td>
</tr>
<tr>
<td>PhP</td>
<td>88</td>
<td>1.42±0.26</td>
<td>10.70±0.80</td>
<td>C</td>
<td>1.16±0.13</td>
</tr>
<tr>
<td>βNADP</td>
<td>95</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>G-6-P</td>
<td>76</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>C</td>
</tr>
</tbody>
</table>

The enzyme activity is reported as percentage inhibition of the activity measured with pNPP as substrate. $K_m$ and $V$ (units/mg of protein) values ± S.D. were calculated as reported. Inhibition type: C, competitive; C/N, mixed competitive/noncompetitive. n.d., not determined.

The effect of temperature on enzyme rate was determined in the 20—50°C range. The enzyme activity was not affected by temperatures below 35°C within 30 min of incubation. At higher temperatures, the reaction was linear up to 10 min and thermal denaturation of the enzyme took place above 45°C (data not shown).

3. Effect of Inhibitors The effects of various compounds on the activity of human liver HMW-ZnAP were tested. The enzyme was insensitive to 20 mm NaF, but was inhibited by t-(−)l-tartrate ($I_{50}=12$ mm). The sulphydryl reactive p-chloromercuribenzoate ($p$-CMB) is a potent enzyme deactivating agent ($I_{50}=0.2$ mm), although iodoacetamide was ineffective at concentrations up to 10 mm. Metal chelating agents such as EDTA ($I_{50}=4$ mm), cysteine ($I_{50}=4$ mm), cyanate ($I_{50}=4$ mm), imidazole ($I_{50}=1$ mm), oxalate ($I_{50}=0.5$ mm) and histidine ($I_{50}=8$ mm) were also inhibitors. Okadaic acid (0.1—10 nm) showed no effect.

4. Effect of pH The pH-dependence of the steadystate kinetic parameters, $K_m$ and $V$, was studied in detail between pH 4.5—9.0. The enzyme lost no activity when incubated for 30 min at these pH values. The data for the $pK_a$ determination were treated according to Dixon and Webb. The pH did not affect $V$ and $K_m$ between pH 5.75—7.50, whereas the values decreased at pH values above 7.50 and below 5.50. The $pK_a$ and $V/K_m$ graphs (Fig. 3) show that the ionization of the enzyme or substrate groups with $pK_a$ values between 5.50—6.00 and 7.25—7.50 plays an important role in catalysis. The former value is similar to the dissociation constant of the substrate pNPP ($pK_a=5.6$): the latter value suggests the involvement of a group in the enzyme with a dissociation constant in the range similar to that of histidine ($pK_a=6.8$). The involvement of a phosphohistidine as an intermediate in the reaction catalyzed by phosphate ester hydrolyzing enzymes has been demonstrated.

5. DEP Inhibition To further investigate the role, if any, of histidine, the DEP inhibition of human liver HMW-ZnAP was studied. The enzyme was inhibited in a time-dependent manner and the inhibition followed pseudo first-order reaction kinetics. The pseudo first-order constant ($k_{inact}$) calculated from the slopes of the lines in Fig. 4, and plotted against DEP concentration shows a straight line (inset of Fig. 4). The second-order rate constant for inactivation was calculated to be $0.435 \times 10^{-3}$ M$^{-1}$ min$^{-1}$. A double-logarithmic plot of the half-times of inactivation against reagent concentration* yields a reaction order of 1.56. The presence of 20 mm phosphate in the incubation medium protected the enzyme treated with 4 mm DEP, suggesting the modification of a residue at the active site. Although DEP is a fairly specific acylating
agent, it has been shown to modify amino acids other than histidines. However, DEP inactivation is reversed by hydroxylamine if the imidazole side-chain of histidine or the phenolic side-chain of tyrosine is modified. The enzyme, inactivated 50% by 4 mM DEP, is only reactivated 15–20% by 0.5 mM hydroxylamine. Although the results of the pH-dependence of the steady-state kinetic parameters seem to be in accord with histidine modification, the partial reactivation with 0.5 mM hydroxylamine suggests that other groups may also be involved in catalysis.

In conclusion, this paper demonstrates the presence of an HMW-ZnAP-type in normal human liver. The enzyme was characterized with respect to some structural, biochemical and kinetic properties. Our results indicate that human liver HMW-ZnAP has different biochemical properties from lysosomal HMW-AP and cytotoxic LMW-AP of the same origin. The enzyme is also distinct from prostatic and platelet-derived AP, as well as from bone tartrate-resistant AP (TRAP).

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